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LOGUES, AND SOME MONOCHLOROPHENOXYACETIC
ACIDS. NEW EXAMPLES OF β OXIDATION

BY STANLEY LEVEY AND HOWARD B. LEWIS

*(From the Department of Biological Chemistry, Medical School,
University of Michigan, Ann Arbor)*

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THE METABOLISM OF PHENOXYACETIC ACID, ITS HOMOLOGUES, AND SOME MONOCHLOROPHENOXYACETIC ACIDS. NEW EXAMPLES OF β OXIDATION

By STANLEY LEVEY* AND HOWARD B. LEWIS

(From the Department of Biological Chemistry, Medical School,
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The biological behavior of benzoic acid and its homologue, phenylacetic acid, has been the subject of many investigations. Phenylacetic acid, like benzoic acid, conjugates with glycine in the organism of the common laboratory animals (dog, cat, rabbit) to form phenaceturic acid, the homologue of hippuric acid (1). Phenoxyacetic acid is a compound which differs from phenylacetic acid in that there is an ether linkage between the benzene ring and the aliphatic side chain ($\text{C}_6\text{H}_5\text{—O—CH}_2\text{—COOH}$). It was of interest to determine the biological behavior of phenoxyacetic acid and some of its higher homologues.

When glycine is fed to the rabbit with phenylacetic acid, the rate of excretion as the conjugation product, phenaceturic acid, is increased. If phenoxyacetic acid were conjugated with glycine, as is phenylacetic acid, the administration of glycine with the acid should increase both the extent of conjugation and the rate of excretion of the conjugate. No evidence of conjugation with glycine was obtained either when the acid was fed alone or with glycine. No evidence of conjugation with glucuronic acid could be obtained. The *o*- and *p*-monochlorophenoxyacetic acids were also studied. The halogen derivatives are of interest in view of the use of the dichloro derivative of phenoxyacetic acid as a weed killer in agriculture. The activity of phenoxyacetic acid and its monochloro derivatives as plant hormones has been studied by Zimmerman (2).

In his studies on biological oxidation of fatty acids (3), Knoop employed fatty acids in which a phenyl group was substituted in the carbon chain at the ω - or terminal carbon atom, and from these observations developed the well known theory of β oxidation. Carter (4) has also used similarly substituted fatty acids to study the mechanism of fatty acid oxidation. The failure of oxidation of phenoxyacetic acid might make this compound of value in the study of the oxidation of its higher homologues, γ -phenoxybutyric and ϵ -phenoxycaproic acids, since β oxidation of these acids should result in the formation of phenoxyacetic acid which escapes further oxidation. These homologues of phenoxyacetic acid were synthesized

* Present address, Wayne County General Hospital, Eloise, Michigan.



and fed to rabbits. The unchanged acids could not be found in the urine, but phenoxyacetic acid could be isolated from the urine by the formation of the S-benzylthiuronium salt. Since phenoxyacetic acid could originate only from the oxidation of the acids fed, the experiments afford new evidence of the β oxidation of fatty acids.

EXPERIMENTAL

Compounds Used—Phenoxyacetic acid and the *o*- and *p*-monochlorophenoxyacetic acids were obtained through the courtesy of Mr. W. M. Alles of the Dow Chemical Company, to whom we wish to express our appreciation of his cooperation. The purity of these acids was established by the determination of their melting point and of their neutral equivalents. γ -Phenoxybutyric acid was synthesized by the method of Bentley, Haworth, and Perkins (5), and ϵ -phenoxycaproic acid by the method of von Braun (6). The ϵ -phenoxycaproic acid, even after three recrystallizations from petroleum ether, melted at 55–56° (uncorrected), a melting point definitely lower than that of 71°, as reported by von Braun.

Since it was anticipated that if phenoxyacetic acid were conjugated with glycine, the conjugated acid, phenoxyacetyl glycine,¹ would be excreted, it was necessary to prepare this acid whose synthesis has not been carried out previously, so far as is known to us. Phenoxyacetyl chloride was prepared by the action of thionyl chloride on phenoxyacetic acid (7). The acid chloride was then coupled with glycine by the usual Schotten-Baumann procedure. No difficulties were encountered in the synthesis. After repeated recrystallization from hot water and drying over phosphorus pentoxide, the acid melted at 115–116° (uncorrected), had a neutral equivalent of 204.6 and 208.2 (theoretical 209), and a nitrogen content of 6.63 per cent (theoretical 6.69 per cent).

In previous studies from this laboratory (8), we have found the S-benzylthiuronium salts of value in the identification of derivatives of fatty acids present in urine. We have prepared these salts of the acids with which we are concerned in the present investigation, applying the conditions for their preparation which are outlined by Donleavy (9). Since, to our knowledge, the preparation and properties of these particular salts have not been described, we are presenting the melting points and nitrogen contents of the derivatives of the pure acids, as well as of those isolated from the experimental urines in Table I. For the isolation and characterization of phenoxyacetic acid and related compounds from urine, the acidified urine has been extracted by ether in a continuous extractor, and the ether has been removed from the extract by evaporation.

¹ The name, "phenoxyaceturic acid," will be used for convenience throughout this discussion in place of phenoxyacetyl glycine. This nomenclature is similar to that of phenaceturic acid for phenylacetyl glycine.

The residual material was neutralized by sodium hydroxide, the sodium salts were dissolved in hot alcohol, and the salts were precipitated as described (8, 9). The derivatives prepared from the ether extracts of urine were usually of satisfactory purity after one recrystallization (8).

TABLE I

Analyses and Melting Points of S-Benzylthiuronium Salts of Phenoxyacetic Acid, o- and p-Monochlorophenoxyacetic Acids Prepared from Pure Acids and Isolated from Experimental Urines

In all the experiments mixed melting points of the salts isolated from the experimental urines and of the salts of the pure acid were determined also. No depression of the melting points were observed. All melting points are uncorrected.

Acid fed		Isolated as acid fed		M.p.	Nitrogen	
(a)	(b)	$\frac{b}{a} \times 100$			Found	Calculated
mg.	mg.		°C.	per cent	per cent	
A. Pure compounds						
Phenoxyacetic			170	8.82	8.83	
<i>o</i> -Chlorophenoxyacetic			159 -159.5	7.99	8.09	
<i>p</i> -Chlorophenoxyacetic			183.5-184	7.99	8.09	
γ -Phenoxybutyric			139 -140			
B. Isolated from urine						
Phenoxyacetic	560	286	51	170		
“	700	340	48	170		
“	600	257	43	170 -171	8.73	8.83
<i>o</i> -Chlorophenoxyacetic	400	216	54	158	8.06	8.09
“	300	133	44	158 -159		
<i>p</i> -Chlorophenoxyacetic	486	193	41	183 -184		
“	300	106	35	183 -184	7.94	8.09
γ -Phenoxybutyric	400	44*	13†	170	8.72	8.83
“	350	40	14†	169		
“	500	160	38†	170	8.73	8.83
ϵ -Phenoxycaproic	420	145*	23†	169	8.62	8.83
“	420	125	20†	170	8.96	8.83

* Carbon and hydrogen determinations made on these salts showed 60.39 and 60.55 per cent of carbon and 5.77 and 5.93 per cent of hydrogen on the respective samples indicated. Theoretical for the salt of phenoxyacetic acid, 60.31 and 5.66 per cent of carbon and hydrogen respectively.

† The salt obtained was the salt of phenoxyacetic acid, as shown by the melting points and analyses. The percentage is calculated as the percentage of the phenoxyacetic acid which could be derived by oxidation of the amount of acid fed.

Methods of Analysis—Since phenoxyacetic acid was a possible urinary component after the administration of phenoxyacetic acid, it was necessary to study methods for its determination. In the Griffith method for the determination of the similar compound, hippuric acid, the acidified urine

is extracted with ether and the nitrogen content of the extract is determined as a measure of the hippuric acid content after the destruction of any extracted urea by the use of hypobromite (10). We have found that phenoxyacetic acid added to normal rabbit urine may be satisfactorily recovered (97.6, 98.1, and 99.0 per cent in three consecutive tests) in this way. It was also desirable to determine total phenoxyacetic acid in urine. The procedure used by Kingsbury and Swanson (11) for the estimation of total benzoic acid, when applied to phenoxyacetic acid, resulted in the formation of some substance (presumably a nitrophenol derivative) which was not completely extracted by the chloroform and, in addition, was only partially reextracted from the chloroform by the salt solution. The yellow color of this material interfered with the titration of the chloroform extract with alkali.

A colorimetric procedure was developed which was based on the oxidative cleavage of phenoxyacetic acid by nitric acid to yield a dinitrophenol. The intensity of the color of the sodium salt of dinitrophenol was measured with the aid of the Evelyn photoelectric colorimeter.

The 6 or 18 hour specimens of rabbit urine were diluted to 100 and 200 ml. respectively, and 5 ml. of this diluted urine were further diluted to 500 ml. 2 ml. of the urine as finally diluted were placed in a micro-Kjeldahl flask calibrated at 12 ml. A glass bead was added and the contents of the flask were reduced nearly to dryness by heating, preferably on a Kjeldahl micro digestion rack. Heating was stopped just before the last drop of liquid disappeared from beneath the bead. 3 ml. of concentrated nitric acid were added and the acid was evaporated by heating on the rack until a small amount of acid remained in the flask. It was necessary to exercise caution at this point, since, if the residue was allowed to become entirely dry, the contents sometimes exploded and low results were obtained. After cooling, 5 ml. of distilled water were added, followed by 3 ml. of 25 per cent sodium hydroxide. After dilution to the mark with distilled water and careful mixing, the intensity of the color which developed immediately was measured in an Evelyn photoelectric colorimeter (Filter 420). The readings were converted to mg. with the aid of a calibration curve, obtained by the use of the above procedure with 2 ml. of normal diluted rabbit urine to which known amounts of phenoxyacetic acid had been added. Aqueous solutions of the acid could not be used in the establishment of the calibration curves, since there was present in rabbit urine some substance, in the presence of which the color of the sodium salt of the dinitrophenol was intensified. By the use of this procedure, it was possible to recover from 93 to 99 per cent of added phenoxyacetic acid. Similar calibration curves were prepared for the monochloro derivatives, for phenoxyacetic acid (which gave, as expected,

essentially the same values as phenoxyacetic acid), and for γ -phenoxybutyric acid. With the monochloro compounds, it was necessary to use fuming nitric acid to split the ether linkage.

Glucuronic acid was determined colorimetrically by the procedure already described from this laboratory (8) with the use of the photoelectric colorimeter. Creatinine was determined as a check on the completeness of the collection of the sample, but the results are not recorded.

Experimental Procedure—Male rabbits of 2 to 3 kilos of body weight, maintained on constant diets of oats and cabbage, served as experimental animals. Urine was collected by gentle pressure on the bladder and was separated into 6 and 18 hour fractions. The sodium salts of the acids in aqueous solution were fed by stomach tubes. Phenoxyacetic acid was first fed at a level of 200 mg. per kilo, but later a somewhat smaller dosage of 100 mg. per kilo was fed. When glycine was also administered, it was given in amounts corresponding to three equivalents of the acid fed.

Since, so far as is known to us, reports of the toxicity of the monochloro derivatives of phenoxyacetic acid are not available, preliminary experiments with these acids were carried out. Male white rats, 200 gm. in weight, received approximately 200, 400, and 800 mg. per kilo orally for 3 consecutive days with no signs of toxicity. The animals consumed their daily food rations completely. Rabbits which received *p*-monophenoxychloroacetic acid at the 200 mg. per kilo level showed a marked hematuria and proteinuria which persisted for approximately 72 hours after the feeding. In subsequent feeding experiments with rabbits, the monochlorophenoxyacetic acids were fed at a level of 125 mg. per kilo (equivalent to 100 mg. of phenoxyacetic acid).

DISCUSSION

The results of the experiments in which phenoxyacetic acid was fed may be summarized as follows. There was no evidence that phenoxyacetic acid was conjugated to a significant degree with either glucuronic acid or with glycine (even when extra glycine in considerable amounts was supplied in the diet). These results, as far as conjugation with glycine to give phenoxyaceturic acid is concerned, are in harmony with the limited earlier and essentially qualitative experiments with man (12, 13) and the dog (13). The excretion of "total" phenoxyacetic acid by the kidneys was rapid, 60 per cent of the amount ingested being excreted within 6 hours (range, 44 to 72 per cent in nine experiments), and 96 per cent in 24 hours (range, 82 to 105 per cent). It is of interest to compare the rate of excretion of phenoxyacetic acid with that of phenylacetic acid in the same species, even though the dose per kilo was significantly greater in the experiments with the latter (1). The excretion of phenylacetic acid, both total

and combined as phenaceturic acid, was slow and the excretion continued over a period of several days.

It was also possible to isolate phenoxyacetic acid from the experimental urines as the S-benzylthiuronium salts² by the procedure already discussed. As shown in Table I, from 43 to 51 per cent of the acid fed could be isolated thus as a derivative of satisfactory purity, as shown by melting points and analyses for nitrogen.

When *o*-monochlorophenoxyacetic acid was fed either with or without glycine, there was no evidence of conjugation, and prompt excretion by the kidneys was observed, *i.e.* in four experiments from 50 to 72 per cent of the acid administered in 6 hours. These results are similar to those obtained with the unsubstituted phenoxyacetic acid and are in sharp contrast to the results obtained after the oral administration of the isomeric *p*-monochlorophenoxyacetic acid. Here also no conjugation was observed, but the excretion in the first 6 hours was so low (13 to 15 per cent of the administered acid in four experiments) that the values approached the lower limits of accuracy of the analytical procedures used. With the dosage used (125 mg. per kilo) there were no signs of toxicity, although the possibility of delayed absorption from the intestine or renal injury as factors in the lower rate of excretion cannot be ruled out. In the 24 hour period the total excretion was essentially the same for both the monochloro acids, an excretion corresponding to 70 to 90 per cent of the amount administered. It was possible to isolate each of these acids from the experimental urines as the SBT salts. The data are presented in Table I.

Since it was shown that phenoxyacetic acid was excreted unchanged, it was of interest to determine whether the higher homologues of phenoxyacetic acid would be excreted unchanged or whether, by oxidation of the side chain, they would be converted to lower homologues or to phenoxyacetic acid itself. When γ -phenoxybutyric acid was fed with or without glycine, the rate of excretion of metabolic products was similar to that observed after phenoxyacetic acid (53 and 58 per cent in 6 hours in two experiments), with no evidence of conjugation with either glycine or glucuronic acid. That oxidation to yield phenoxyacetic acid had occurred was shown by the isolation of the SBT salt of phenoxyacetic acid from the experimental urines. As shown in Table I, the amounts of the salt isolated in three experiments were equivalent to 13, 14, and 38 per cent of the theoretical amount of phenoxyacetic acid which could be obtained by β oxidation of the γ -phenoxybutyric acid fed.

After ϵ -phenoxy-caproic acid had been fed to two rabbits, 20 and 23 per cent of the theoretical amount of the SBT salt of phenoxyacetic acid was obtained from the urine. Melting points, mixed melting points, and

² For convenience, these salts will be referred to hereafter as SBT salts.

analyses for nitrogen were satisfactory (Table I). However, as added proof, one sample of the phenoxyacetic acid salt isolated after the administration of γ -phenoxybutyric acid and one sample obtained after ϵ -phenoxy-caproic acid were analyzed for carbon and hydrogen, as shown in the foot-notes to Table I. All the evidence indicates clearly that the oxidation, presumably β oxidation, of the acids has occurred, and that the product of oxidation, phenoxyacetic acid, is excreted in the urine. Since the preparation of the SBT salts of other acids from urine has been estimated to yield from 50 to 60 per cent of the theoretical amount (8), it is believed that the amount of phenoxyacetic acid actually present in the urine represents a significant proportion of the acid fed.

Although no conjugation of phenoxyacetic acid with glycine was observed, it was of interest to study the fate of the potential conjugation product, phenoxyaceturic acid, which we had synthesized as already described. The acid was administered orally or subcutaneously to rabbits in amounts equivalent to 100 mg. of phenoxyacetic acid per kilo and the total phenoxyacetic acid and the phenoxyacetic acid as phenoxyaceturic acid were determined in the 6 and 18 hour urines. The difference between total and conjugated phenoxyacetic acid was an index of the amount of phenoxyaceturic acid hydrolyzed. With two exceptions (one oral and one subcutaneous administration), significant hydrolysis was observed, a hydrolysis which was greater in the 18 hour samples than in the 6 hour. That this hydrolysis was not due to the activity of the intestinal microflora was shown by the observation that in four experiments with subcutaneous injection hydrolysis was observed in three of the four urines collected after 6 hours and in all four of the 18 hour specimens. From the urine of one animal which had received 500 mg. of phenoxyaceturic acid orally, the equivalent of 108 mg. of phenoxyacetic acid was isolated as the SBT salt. This corresponded to about 30 per cent of the phenoxyacetic acid which could be obtained by complete hydrolysis of the phenoxyaceturic acid fed. The melting point, mixed melting point, and nitrogen content (8.75 per cent) corresponded to those of known samples of the SBT salt of phenoxyacetic acid.

The hydrolytic cleavage of phenaceturic acid observed was not due to changes in the urine after voiding. When urine samples containing phenaceturic acid were incubated at room temperature, hydrolysis was slight, less than 6 per cent in 24 hours. A similar study of hippuric acid in urine showed a hydrolysis of more than 40 per cent in two experiments.

Histozyme, a non-specific enzyme, which effects the hydrolysis of hippuric acid, is known to occur in certain animal tissues, notably the kidney. So (14) has observed that phenaceturic acid was readily hydrolyzed by a purified histozyme preparation from pig liver, less readily by a similar

preparation from pig kidney. We have prepared partially purified histozyme (acetone-dried preparations) from rabbit kidney by the method of So (14) and have studied its action on hippuric and phenoxyacetic acid in parallel experiments.

Hippuric or phenoxyacetic acid was determined by the use of the Griffith method (10) before and after incubation. The decrease after incubation represented the amount hydrolyzed. The details are presented

TABLE II

Hydrolysis of Hippuric and Phenoxyacetic Acids by Histozyne of Rabbit Kidney

Test solutions included 25 ml. of phosphate buffer (pH 6.8), 200 mg. of enzyme powder,* and the amount of substrate indicated. The mixed materials were diluted to 50 ml. with distilled water. A small crystal of thymol was added, and the solution was incubated for 24 hours at 36–37°. Blank values have been subtracted.

Experiment No.	Time	Substrate		Hydrolysis
	hrs.		mg.	per cent
1	0	Hippuric acid	108.0	
	24	" "	88.2	18.3
2	0	Phenoxyacetic acid	101.5	
	24	" "	32.6	68.9
3	0	Hippuric acid	126.4	
	24	" "	121.3	3.25
4	0	Phenoxyacetic acid	100.8	
	24	" "	66.7	33.8
5	0	Hippuric acid	143.4	
	24	" "	142.2	0.9
6	0	Phenoxyacetic acid	147.4	
	24	" "	100.3	32.1
7	0	Hippuric acid	131.1	
	24	" "	122.8	7.1
8	0	Phenoxyacetic acid	75.3	
	24	" "	47.3	37.2

* Two histozyme preparations were used: Preparation 1 in Experiments 1 and 2, and Preparation 2 in the other experiments.

in Table II. Preparation 1 of the kidney powder was more effective in the hydrolysis of both hippuric and phenoxyacetic acid than was Preparation 2, which hydrolyzed hippuric acid very slightly. However, in each of the four sets of experiments, the extent of hydrolysis of phenoxyacetic acid was clearly greater than was that of hippuric acid. The data suggest that the hydrolysis of phenoxyacetic acid, observed after both oral and parenteral administration, may be related to the activity of the histozyme of the kidney. It is, of course, possible that similar enzymatic activity may be associated with other organs.

SUMMARY

The S-benzylthiuronium salts of phenoxyacetic acid and some of its derivatives and homologues have been prepared and their properties described. These salts have been useful for the identification of the phenoxy acids and their products of metabolism in urine. Phenoxyacetyl-glycine ("phenoxyaceturic" acid) has been synthesized. When this compound was fed or injected into rabbits, some hydrolysis occurred, as evidenced by the excretion of phenoxyacetic acid in the urine. The presence of an enzyme which hydrolyzed phenoxyaceturic acid more readily than hippuric acid was demonstrated in "histozyme" preparations from rabbit kidneys (acetone-dried tissue).

When phenoxyacetic and *o*- and *p*-monochlorophenoxyacetic acids were fed to rabbits, they were excreted in the urine. No evidence of conjugation with either glycine (phenoxyaceturic acids) or glucuronic acid was obtained.

When γ -phenoxybutyric acid and ϵ -phenoxy-caproic acid were fed, only phenoxyacetic acid was isolated from the urine. This is further evidence in support of the Knoop theory of β oxidation of fatty acids, since the phenoxyacetic acid is believed to be derived from the higher homologues by β oxidation.

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